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Icariin reduces α-synuclein over-expression by promoting α-synuclein degradation

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Abstract The objectives of this study are to investigate the effects of icariin (a main component extracted from *Epimedium*) on over-expression of α-synuclein and to explore the underlying mechanisms. APPV717I transgenic (Tg) mice and A53T α-synuclein-transfected PC12 cells were used in this study. The content of α-synuclein mRNA was determined by reverse-transcription PCR (RT-PCR). Western blotting and immunohistochemistry were used to detect the protein expression of α-synuclein, parkin, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), and heat shock protein 70 (HSP70). In 10-month-old APP Tg mice, α-synuclein expression was increased, and the expression of Parkin, UCH-L1, and HSP70 was decreased in the hippocampus. Intragastrical administration of icariin (30 and 100 μmol/kg) for 6 months (from 4 to 10 months old) decreased α-synuclein expression and increased the expression of Parkin, UCH-L1, and HSP70 in the hippocampus of APP Tg mice. Incubation of icariin (40 and 80 μM) with A53T α-synuclein-transfected PC12 cells for 24 h showed no difference in the expressions of α-synuclein mRNA among model group and icariin-treated groups, but decreased α-synuclein protein expression in both monomer and tetramer. Along with the downregulation of α-synuclein, icariin (40 and 80 μM) elevated the expression of Parkin, UCH-L1, and HSP70 in A53T α-synuclein-transfected cells. Icariin inhibited the over-expression of α-synuclein both in vivo and in vitro. The mechanism of icariin may be related to upregulate Parkin and UCH-L1 expression in ubiquitin-proteasome system and HSP70 in molecular chaperone, thus enhancing the degradation of α-synuclein. It is suggested that icariin may have the potential to treat Alzheimer's disease (AD) and other synucleinopathies.

Keywords Icariin · α-synuclein · Ubiquitin-proteasome system · Heat shock protein 70 · Alzheimer's disease · Synucleinopathies

Abbreviations

AD	Alzheimer's disease
HSPs	Heat shock proteins
NACP	The precursor of the non-amyloid-beta component
Tg	APPV717I transgenic
UCH-L1	Ubiquitin carboxy-terminal hydrolase L1
UPS	Ubiquitin-proteasome system

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Introduction

Alzheimer's disease (AD) is characterized by the accumulation of senile plaques and neurofibrillary tangles in the brain. The senile plaques contain not only β-amyloid (Aβ) but also the non-amyloid-β component (Ueda et al. 1993). The precursor of the non-amyloid-β component of plaques (NACP) is also known as α-synuclein, which is a presynaptic terminal molecule and accumulates in AD senile plaques (Iwai 2000). α-Synuclein contains 140 amino acid residues and plays an important role in synaptic plasticity and neurotransmission under normal conditions (Gerlach et al. 2012). Aberrant accumulation of α-synuclein into aggregates has been identified as a pathological hallmark of synucleinopathies including Parkinson's disease and dementia with Lewy body (DLB) (Gentile et al. 2008; Stefanis 2012). Up to 60 % of AD cases exhibit significant synucleinopathology in addition to plaques and tangles (Hamilton 2000). An increasing body of evidence suggests that over-expression and aggregation of α-synuclein may play pathogenic roles in synaptic dysfunction, cytotoxicity, and AD (Lewis et al. 2010). In our previous study, we reported that α-synuclein mRNA and protein expression were increased in a time-dependent manner in the hippocampus of APPV717I transgenic mice at the ages of 4, 10, and 16 months, and α-synuclein aggregation occurred at the age of 16 months (Zhang et al. 2013). We suggest that, besides increased Aβ and amyloid plaques, over-expression and aggregation of α-synuclein in the hippocampus might partially account for cognitive impairment in this APP Tg mouse model of AD (Zhang et al. 2013). The role of α-synuclein should be considered when developing new therapeutic strategies to target AD pathogenesis.

Icariin ($C_{33}H_{40}O_{15}$; molecular weight 676.67), a major active component extracted from the traditional Chinese herb *Epimedium*, has been reported to have extensive pharmacological effects (Chen et al. 2005). Urano et al. reported that administration of icariin improved spatial memory impairment in transgenic mouse AD model (5xFAD) and attenuated neurite atrophy in an in vitro cell model induced by $Aβ_{1-42}$ (Urano and Tohda 2010). In our previous study, we found that icariin reduced the Aβ burden and amyloid plaque deposition in the hippocampus of APPV717I transgenic mice (Zhang et al. 2014). Since α-synuclein is the precursor of the non-amyloid-β component of AD senile plaques and there are some crosstalks among various neurodegenerative diseases, we speculated that icariin may also influence α-synuclein and this effect may be a novel mechanism of icariin in AD therapy.

In order to verify this hypothesis, we investigated the pharmacological effects of icariin on α-synuclein expression and aggregation and explored the underlying mechanisms by using APPV717I transgenic mice and A53T α-synuclein-transfected PC12 cells in the present study.

Materials and methods

Drug

Icariin was purchased from the National Institute for Food and Drug Control (Beijing, China), with a molecular weight of 676.65 ($C_{33}H_{40}O_{15}$) and 99 % purity as determined by a high-performance liquid chromatography assay. For animal experiments, powder of icariin was dissolved in distilled water at concentrations of 30 and 100 μM. For cell experiments, a stock solution of 100 mM icariin was prepared in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) and stored at −20 °C. The stock solution was diluted to the appropriate concentrations with culture medium. The final concentration of DMSO was less than 0.1 % (vol/vol) (Hong et al. 2013).

Animals

The APPV717I transgenic mice and their wild-type (WT) littermates were obtained from the Institute of Experimental Animal, Chinese Academy of Medical Sciences (Beijing, China). They are bred on a C57/BL6 strain and express high levels of human APP751 containing the London (V717I) mutation under the platelet-derived growth factor promoter (Zhang et al. 2003), which results in a marked increase of Aβ1-42 production (Sturchler-Pierrat et al. 1997). Animal housing and all experimental procedures followed the requirements of the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. Animals were housed under a 12/12-h dark/light cycle and in standard pathogen-free conditions. They had free access to food and water throughout the entire experiment.

After 1-week acclimatization to their home cage, the APPV717I Tg mice were randomly divided into three groups: APP Tg, APP Tg + icariin 30 μmol/kg, and APP Tg + icariin 100 μmol/kg. They received daily gavages of dissolved icariin or an equal volume of distilled water (0.1 ml/10 g weight) for 6 months (from 4 to 10 months old). Each group consisted of 12 mice (half female and half male). The same number of wild-type littermates and wild-type littermates with 100 μmol/kg icariin administration for 6 months served as normal controls and normal drug controls, respectively.

Cell culture

A53T α-synuclein-transfected PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 5 % fetal bovine serum (FBS), 10 % equine serum (Gibco), and 100 U/ml penicillin and streptomycin (Gibco BRL, Gaithersburg, MD, USA) at 37 °C in a 5 % CO_2 humidified incubator. Cells were passaged per 3 days. All experiments were conducted in triplicate to ensure reproducibility.

Western blot

The hippocampi from five individual mice per group were homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 0.5 mM ethylene glycol tetraacetic acid, 150 mM NaCl, and 1 % protease inhibitor cocktail (Sigma, USA). Western blot analysis was performed as described previously (Vivacqua et al. 2009). Proteins were separated using 15 % sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto a polyvinylidene fluoride membrane, and reacted with primary antibodies against α-synuclein (Abcam ab1903), HSP70, UCH-L1, and Parkin (Santa Cruz) overnight at 4 °C, respectively, followed by a horseradish peroxidase-conjugated goat-anti-mouse or goat-anti-rabbit IgG secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL system; Santa Cruz Biotechnology, CA, USA) and exposed to MR X-ray film (Kodak, USA).

Immunohistochemistry

Three mice in each group were sacrificed and immediately perfused through the heart with 0.9 % NaCl, which was followed by 4 % paraformaldehyde in 0.1 M phosphate buffer for 10 min. Brains were postfixed by immersion for 4 days and then cryoprotected in 30 % sucrose. Brains were rapidly frozen in OCT embedding medium, and frozen sections with a thickness of 20 μm were taken through the hippocampus and processed in free-floating conditions. Immunohistochemical procedures were performed as described previously (Yu et al. 2007) with slight modifications. Sections were treated with 0.1 M phosphate-buffered saline containing 0.3 % Triton X-100 (PBST) for 4 days and first incubated with the primary antibodies against α-synuclein (anti-α-synuclein monoclonal antibody 3D5 was kindly provided by Dr. Shun Yu because this antibody recognizes amino acids 115–121 of α-synuclein both in synapses and nuclei, Yu et al. 2007) overnight at 4 °C. HSP70 antibody from Santa Cruz was used in this experiment. The sections were then incubated with the second biotinylated goat anti-mouse IgG antibody at room temperature for 2 h and finally with horseradish-conjugated streptavidin at room temperature for 1 h. Peroxidase activity was revealed by 0.02 % 3,3′-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.005 % H_2O_2 and 0.3 % nickel ammonium sulfate. The stained sections were mounted on glass slides, dehydrated, cleared, and coverslipped.

Reverse transcription PCR

A previously published protocol of reverse-transcription PCR (RT-PCR) (Vila et al. 2000) was followed to detect the expression of α-synuclein mRNA. Briefly, TRIzol (Invitrogen, Carlsbad, CA, USA) was used to isolate RNA from the cells of different treatments. RNA (5 μg) was reverse transcribed for 1 h at 42 °C by using an oligo-dT15 primer from the Reverse Transcription System (Promega, Madison, WI, USA). The resulting cDNA was amplified by PCR that used sequence-specific primers for α-synuclein (data from the National Center for

Biotechnology Information Database, USA). The primers (Shanghai Bio-engineering Company, China) designed for α-synuclein (forward primer: 5′-ATAAGAATGCGGCCGCATGGATGTATTCATGAAAG-3′, reverse primer: 5′-CCGCTCGAGGCTTCAGGTTCGTAGTCTTGA-3′) resulted in a PCR product of 444 base pairs (bp). Results for each individual cDNA were normalized by dividing the relative amount of each transcript by the relative amount of actin transcript from the same experiment. All ingredients, except primers, were the same for all reverse-transcription PCRs (RT-PCRs). The products were visualized after electrophoresis on a 2 % agarose gel containing ethidium bromide.

Statistical analysis

Data were represented as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) followed by a post hoc Fisher's least-significant difference test. A value of $P < 0.05$ was considered significant.

Results

Effect of icariin on α-synuclein in hippocampus of APP transgenic mice

Figure 1a shows the distribution and amount of α-synuclein-positive inclusions in hippocampi of the different groups with immunohistochemical staining. The α-synuclein-positive marker was presented as brown granule. Compared with the age-matched wild-type littermates, α-synuclein-positive cells were significantly increased with deep staining in the hippocampal area of 10-month-old APP Tg(+) mice. The icariin-treated Tg(+) mice showed less α-synuclein-positive cells and lighter staining in the hippocampus compared with Tg(+) model mice (Fig. 1a).

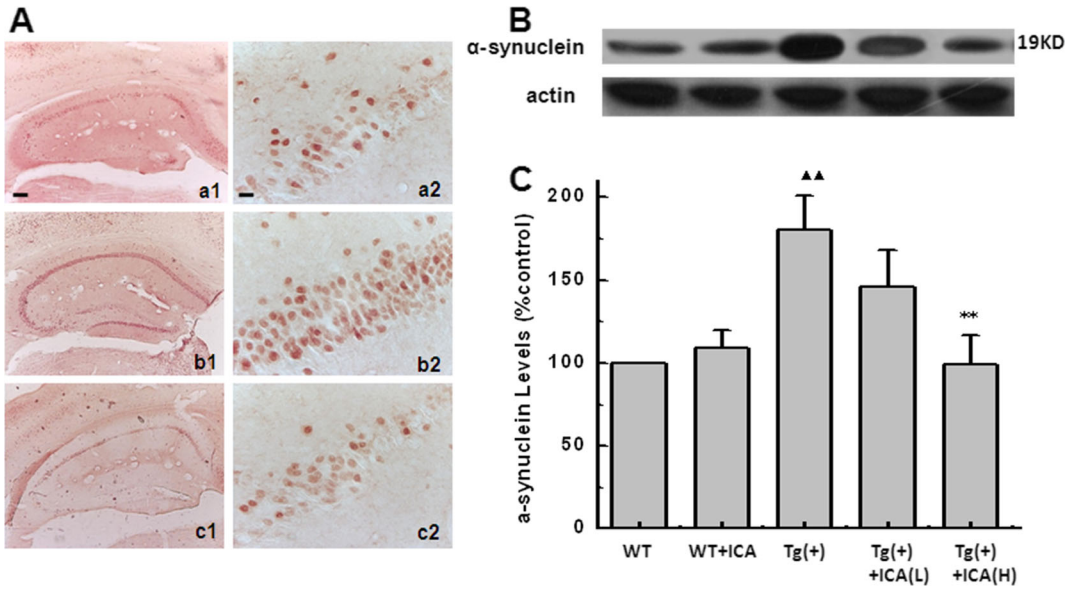

Fig. 1 Effect of icariin on α-synuclein in hippocampus of APP transgenic mice. Immunohistochemical staining of α-synuclein reflect the effect of ICA on α-synuclein expression in 10-month-old APPV717I Tg mouse hippocampal (**a**). *a* Control WT mice; *b* APP Tg model mice; *c* intragastric ICA administration (100 μmol/kg) to APP Tg mice from 4 to 10 months of age. *1* Whole hippocampus (*scale bar* = 200 μm); *2* hippocampal CA2 region at a higher magnification (*scale bar* = 20 μm). Representative bands obtained by Western blotting analysis of α-synuclein in the hippocampus of all experiment groups (**b**). The graph represents the semiquantitative analysis of bands normalized with β-actin density in each sample, respectively (**c**). Values represent the mean value of α-synuclein in hippocampal tissue ($n = 5$); *error bars* represent standard deviation of the mean. *WT*, control wild-type mice; *Tg*, transgenic mice; *ICA(L)*, icariin 30 μmol/kg; *ICA(H)*, icariin 100 μmol/kg; $^{▲▲}P < 0.01$ versus WT group, $^{**}P < 0.01$ versus Tg(+) group

The result from Western blot assay showed that the level of α-synuclein in the hippocampus was significantly increased in the Tg(+) mice ($P < 0.05$). After intragastrical administration of icariin at the dose of 100 μmol/kg for 6 months, the level of α-synuclein was obviously reduced compared with the Tg(+) model group ($P < 0.01$) (Fig. 1b, c).

Effect of icariin on Parkin and UCH-L1 in the hippocampus of APP transgenic mice

Ubiquitin-proteasome system (UPS) has been suggested to contribute to α-synuclein's degradation (McNaught and Jenner 2001). Parkin, a 53-kDa cytosolic protein, is a well-characterized ubiquitin E3 ligase which attaches polyubiquitin chains (at K48 or K63) to known substrates that are targeted for UPS degradation (Shimura et al. 2000; Moore 2006). Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) plays a central role in the UPS, by acting as a hydrolase for ubiquitin chains destined for degradation within the proteasome (Osaka et al. 2003). In our study, Western blot analysis was performed to investigate the key proteins in UPS. Figure 2 shows that the expression of both Parkin and UCH-L1 was significantly reduced in the hippocampus of APP Tg(+) mice at the age of 10 months ($P < 0.05$). Administration of icariin at the dose of 100 μmol/kg to Tg(+) mice obviously increased the expression of Parkin and UCH-L1 ($P < 0.05$), which was recovered to the level similar to that in the wild-type littermates (Fig. 2).

Effect of icariin on HSP70 in the hippocampus of APP transgenic mice

Besides UPS, heat shock proteins (HSPs), a group of important molecular chaperones, also prevent protein misfolding and aggregation and promote the degradation of aberrantly accumulating misfolded proteins (Kilpatrick et al. 2013). Particularly, the 70-kDa heat shock protein (HSP70) is shown to interact with α-synuclein to inhibit the formation of toxic conformations of α-synuclein and to protect the cells from α-synuclein-induced toxicity (Danzer et al. 2011; Kilpatrick et al. 2013). In the present study, immunohistochemical staining and Western blot assay were used to detect the expression of HSP70. The results showed that HSP70 expression was obviously decreased in the hippocampus of APP Tg(+) mice ($P < 0.01$). Treatment of icariin at the dose of 100 μmol/kg significantly elevated HSP70 expression compared with the Tg(+) mold mice ($P < 0.05$) (Fig. 3).

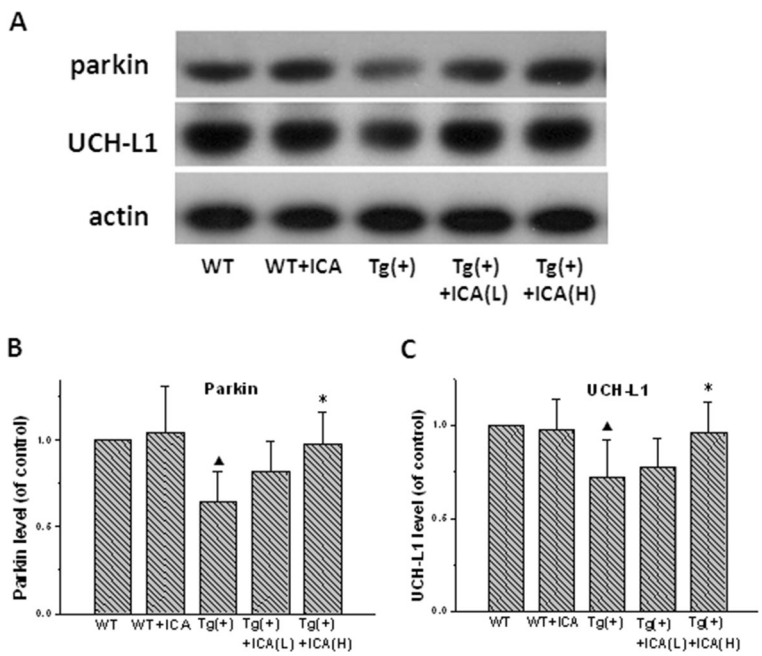

Fig. 2 Effect of icariin on Parkin and UCH-L1 in the hippocampus of APP transgenic mice. Representative bands obtained by Western blotting analysis of Parkin and UCH-L1 in the hippocampus of all experiment groups (**a**). The graphs represent the semiquantitative analysis of Parkin (**b**) and UCH-L1 (**c**) bands normalized with β-actin in each sample, respectively. Values represent the mean value of each protein in hippocampal tissue ($n = 5$); *error bars* represent standard deviation of the mean. *WT*, control wild-type mice; *Tg*, transgenic mice; *ICA(L)*, icariin 30 μmol/kg; *ICA(H)*, icariin 100 μmol/kg; ▲$P < 0.05$ versus WT group, *$P < 0.05$ versus Tg(+) group

Effect of icariin on α-synuclein in A53T α-synuclein-transfected cells

PC12 cells transfected with A53T α-synuclein were used to investigate the direct effects of icariin on α-synuclein. Different dosages of icariin (5~80 μM) were incubated with A53T α-synuclein-transfected cells for 24 h. The mRNA expression of α-synuclein was detected by RT-PCR, and protein level of α-synuclein was detected by Western blot assay. Compared with the model cells, the expression and aggregation of α-synuclein protein (monomer and tetramer) were significantly declined in A53T cells incubated with icariin at the doses of 40 and 80 μM for 24 h ($P < 0.05$, Fig. 4a, b); however, the expression of α-synuclein mRNA was not changed after icariin treatment (Fig. 4c, d).

Effect of icariin on Parkin, UCH-L1, and HSP70 in A53T α-synuclein-transfected cells

The results displayed that the treatment of icariin at the doses of 40 and 80 μM for 24 h significantly elevated the protein expression of Parkin, UCH-L1, and HSP70 in A53T a-synuclein-transfected PC12 cells ($P < 0.05$) (Fig. 5).

Discussion

The APPV717I transgenic mice that we used in this study can mimic the Aβ deposition and senile plaque formation in AD brains (Sturchler-Pierrat et al. 1997) and are generally accepted as an animal model of AD. In our previous study, APP transgenic mice also showed an increased expression of α-synuclein (Zhang et al. 2013), which is the precursor of the non-amyloid-beta component of plaques (Kallhoff et al. 2007). We discovered that icariin reduced the Aβ content and amyloid plaque deposition in the hippocampus of APPV717I transgenic mice (Zhang et al. 2014). In the present study, we found that icariin antagonized α-synuclein over-expression in an AD-like animal model for the first time. In order to further observe the direct effect of icariin on α-synuclein, we used A53T α-synuclein-transfected PC12 cells and found that icariin

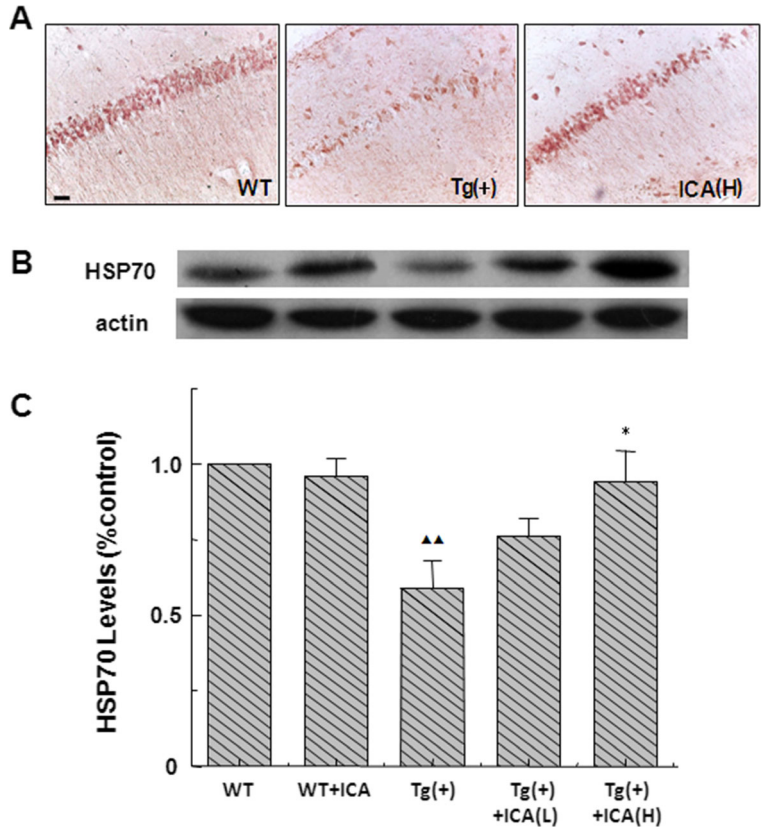

Fig. 3 Effect of icariin on HSP70 in the hippocampus of APP transgenic mice. Immunohistochemical staining of HSP70 reflect the effect of ICA on HSP70 expression in 10-month-old APPV717I Tg mouse hippocampal CA2 region (*scale bar* = 40 μm) (**a**). *WT*, control wild-type mice; *Tg(+)*, transgenic model mice; *ICA(H)*, model mice with intragastric ICA administration (100 μmol/kg) from 4 to 10 months of age. Representative bands obtained by Western blotting analysis of HSP70 in the hippocampus of all experiment groups (**b**). The graph represents the semiquantitative analysis of bands normalized with β-actin density in each sample, respectively (**c**). Values represent the mean value of HSP70 in hippocampal tissue ($n = 5$); *error bars* represent standard deviation of the mean. *WT*, control wild-type mice; *Tg*, transgenic mice; *ICA(L)*, icariin 30 μmol/kg; *ICA(H)*, icariin 100 μmol/kg; ▲▲$P < 0.01$ versus WT group, *$P < 0.05$ versus Tg(+) group

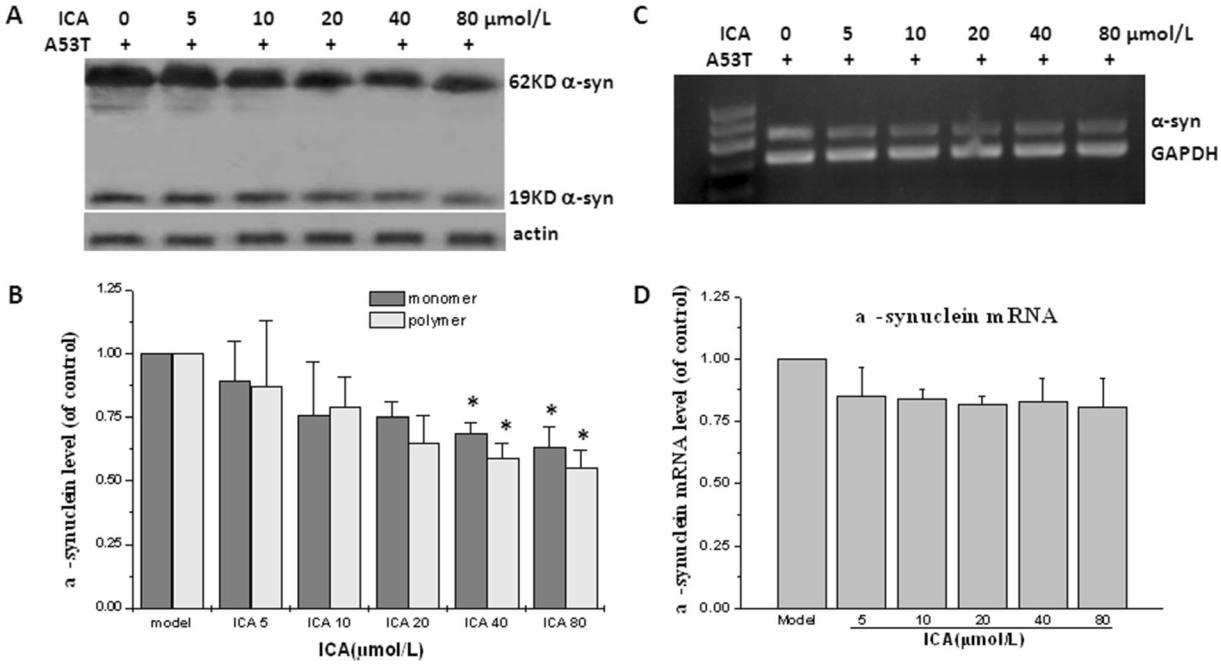

Fig. 4 Effect of icariin on α-synuclein in A53T α-synuclein-transfected PC12 cells. Representative bands obtained by Western blot analysis of α-synuclein in A53T α-synuclein-transfected PC12 cells (**a**). The graph represents the semiquantitative analysis of bands normalized with β-actin density in each sample, respectively (**b**). Representative bands obtained by RT-PCR analysis of α-synuclein in A53T α-synuclein-transfected PC12 cells (**c**). The graph represents the semiquantitative analysis of bands normalized with GAPDH density in each sample, respectively (**d**). Values represent the mean value of α-synuclein in all experimental repeats ($n = 3$); *error bars* represent standard deviation of the mean. Model, A53T α-synuclein-transfected PC12 cells; ICA, different final dosages of icariin; $^*P < 0.05$ versus model group

decreased not only the over-expressions but also the aggregations of α-synuclein in this cell model. However, icariin did not inhibit the expression of α-synuclein mRNA, suggesting that icariin's regulatory effect was not at a transcriptional level, but might be related with the degradation of α-synuclein.

Unfolded or misfolded proteins can be eliminated via the UPS through a series of enzymatic reactions involving ubiquitin (Ub)-activating enzymes (E1), Ub-conjugating enzymes (E2), and Ub-protein ligases (E3) (Hershko et al. 2000). UPS has been suggested to contribute to α-synuclein's degradation (McNaught and Jenner 2001). Although the manner of α-synuclein degradation in neurons remains contentious, drugs target to the protein degradation pathway may be a prospective treatment strategy for neurodegenerative diseases (Xilouri et al. 2013; Jiang and Chen 2012). Parkin, a 53-kDa cytosolic protein, is a well-characterized ubiquitin E3 ligase which attaches polyubiquitin chains (at K48 or K63) to known substrates that are targeted for UPS degradation (Shimura et al. 2000; Moore 2006). Parkin may play a neuroprotective role by contributing to the proteasomal clearance of α-synuclein, thus attenuating its toxicity (Petrucelli et al. 2002). Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) is an abundant, neuron-specific protein. It plays a central role in the UPS, by acting as a hydrolase for ubiquitin chains destined for degradation within the proteasome, as an ubiquitin ligase for the Lys63 residue of the ubiquitin molecule, and by being involved in the maintenance of free ubiquitin monomers levels necessary for the ubiquitination process (Osaka et al. 2003). In the present study, APP V717I transgenic mice displayed a decrease in the expression of Parkin and UCH-Ll in the hippocampus, and the treatment of icariin significantly elevated Parkin and UCH-Ll levels. This effect of icariin was further confirmed in A53T α-synuclein-transfected PC12 cells. The results suggest that icariin enhances the degradation of excessive and abnormal aggregated α-synuclein through upregulating UPS.

HSPs, a group of important molecular chaperones, protect cells from proteotoxic stress by preventing protein misfolding and aggregation and promoting the degradation of aberrantly accumulating misfolded proteins (Kilpatrick et al. 2013). Particularly, the 70-kDa heat shock protein (HSP70) is shown to interact with α-synuclein either

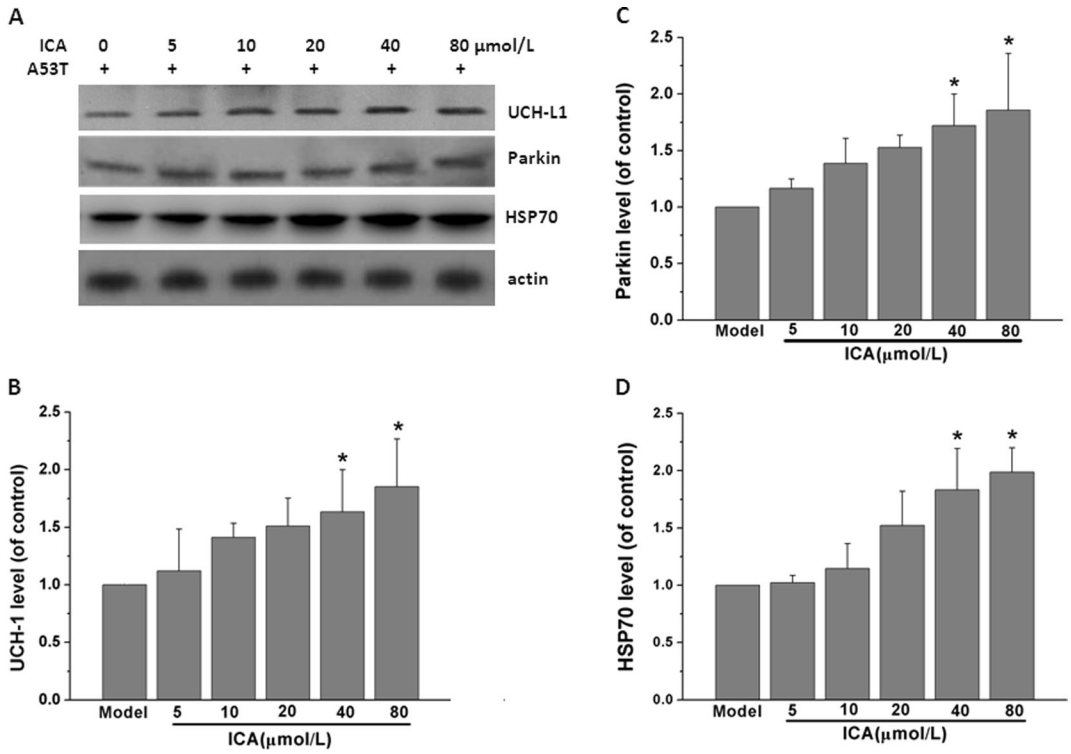

Fig. 5 Effect of icariin on UCH-L1, Parkin, and HSP70 in A53T α-synuclein-transfected cells. Representative bands obtained by Western blot analysis of UCH-L1, Parkin, and HSP70 in A53T α-synuclein-transfected PC12 cells (**a**). The graph represents the semi-quantitative analysis of UCH-L1 (**b**), Parkin (**c**), and HSP70 (**d**) bands normalized with β-actin density in each sample, respectively. Values represent the mean value of each protein in all experimental repeats ($n = 3$); *error bars* represent standard deviation of the mean. Model, A53T α-synuclein-transfected PC12 cells; ICA, different final dosages of icariin; $^*P < 0.05$ versus model group

intracellular or extracellular, to inhibit the formation of toxic conformations of α-synuclein, and to protect the cells from α-synuclein-induced toxicity (Danzer et al. 2011; Kilpatrick et al. 2013). Therefore, HSP70 is suggested to be a novel therapeutic target to antagonize α-synuclein-induced toxicity in neurodegenerative diseases. In the present study, APP transgenic mice showed a decrease in the expression of HSP70 in the hippocampus, and the treatment of icariin obviously increased HSP70 expression. In A53T α-synuclein-transfected PC12 cells, icariin can directly enhance HSP70 expression. This may be a novel mechanism by which icariin decreases the over-expression and aggregation of α-synuclein.

Conclusion

Taken together, our present study indicates for the first time that icariin inhibited the over-expression of α-synuclein in the hippocampus of AD-like APP transgenic mice and in A53T α-synuclein- transfected PC12 cells. Icariin's regulatory effect is not at a transcriptional level, but related with the degradation of α-synuclein. Upregulation of Parkin and UCH-Ll in ubiquitin-proteasome system and HSP70 in the molecular chaperone is the underlying mechanism of icariin to enhance the degradation of α-synuclein. Our findings suggest that icariin may have the potential to treat AD and other synucleinopathies.

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Conflict of interests The authors declare that they have no competing interests.

Authors' contributions LL and LZ conceptualized the study; LZ, CS, JC, YL, and YL-L carried out the experiments; LZ and CS

conducted the analyses and wrote the manuscript. All authors read and approved the final manuscript.

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